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Identification of an Initiator-like Element within the HTLV-I Promoter

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Using the 5′ long terminal repeat (LTR) as its only promoter, the HTLV-1 provirus generates a single RNA transcript that undergoes differential splicing to express the various viral proteins. Examination of sequence near the transcription start site revealed an element resembling a transcriptional initiator (Inr) at position −8 to −15 in addition to the canonical TATA box at −25. To elucidate basal control of HTLV-I gene expression, functional traits of this element were examined. It specifically bound a protein complex, the mobility of which was altered by antibody to serum response factor, and independently mediated reporter gene expression. Mutating the Inr in a minimal construct reduced basal transcription, whereas mutation of the element within the context of the complete LTR left basal transcription unaffected. Presence of the element influenced transcription start site choices. Exhibiting many characteristics of an Inr, this element may play an important role in regulating HTLV-I gene expression *in vivo,* particularly during the long clinical latency period prior to development of HTLV-I-induced disease.

Key Words: initiator element; HTLV-1; promoter; SRF; tax; transcription.

INTRODUCTION

Human T cell leukemia virus type I (HTLV-I) is the etiologic agent of two diseases in humans: adult T-cell leukemia (ATL) and tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM) (Poiesz *et al.,* 1980; Gessain *et al.,* 1985; Osame *et al.,* 1987; Yoshida *et al.,* 1982). HTLV-I-induced disease is characterized by a long period of clinical latency that can span two or more decades. During this latent period, viral gene expression is low to undetectable in infected lymphocytes.

HTLV-I encodes a transcriptional activator, Tax, which activates both viral and cellular gene expression through indirect association with responsive elements. Tax is, moreover, the transforming protein of HTLV-I (Yamaoka *et al.,* 1992; Grassmann *et al.,* 1989, 1992; Tanaka *et al.,* 1990). Because of these critical roles of Tax in viral replication and cellular transformation, characterizing the regulatory elements that control Tax expression is important.

HTLV-I transcription is regulated by elements located in the U3 region of the 5′ proviral long terminal repeat (LTR). Mechanisms of basal transcription regulation of the LTR remain relatively undefined (Barnhart *et al.,* 1997; Andrews *et al.,* 1997; Newbound *et al.,* 1999; Kashanchi *et al.,* 1994). A major HTLV-I transcription start site has been mapped (Piras *et al.,* 1994) to ~25 bp downstream from a consensus TATA box. Site-directed mutation of the TATA box in the HTLV-I LTR, however, had little effect on the basal level of viral transcription (Nicholas and Nevins, 1991), suggesting that the HTLV-I promoter may contain an element other than the TATA box that can direct transcription.

One such element that has been identified in a variety of promoters is an initiator, a transcription control element capable of directing specific transcription initiation by RNA polymerase II. Specifically, initiators can determine the transcription start site in promoters that lack a TATA box or enhance transcription initiation in promoters that contain a TATA box (Javahery *et al.,* 1994). Unlike TATA boxes, which initiate transcription ~30 bases downstream, initiators most commonly direct transcription from an internal residue (O'Shea-Greenfield and Smale, 1992; Javahery *et al.,* 1994; Lo and Smale, 1996).

We have identified a sequence within the HTLV-I LTR that possesses sequence similarity to known Inr elements. This element is capable of both specifically binding a cellular protein and modulating activity of the viral promoter. Based on these results we suggest that this element is an initiator-like element, which may be used to regulate viral gene expression under specific environmental conditions.

RESULTS

The HTLV-I promoter contains a sequence similar to an initiator element

During the long period of clinical latency, HTLV-I gene expression is usually low to undetectable. Since expression of viral gene products such as Tax plays an important role in the development of disease, it is necessary to identify elements within the viral promoter that regulate basal transcription. Visual inspection of the basal pro-

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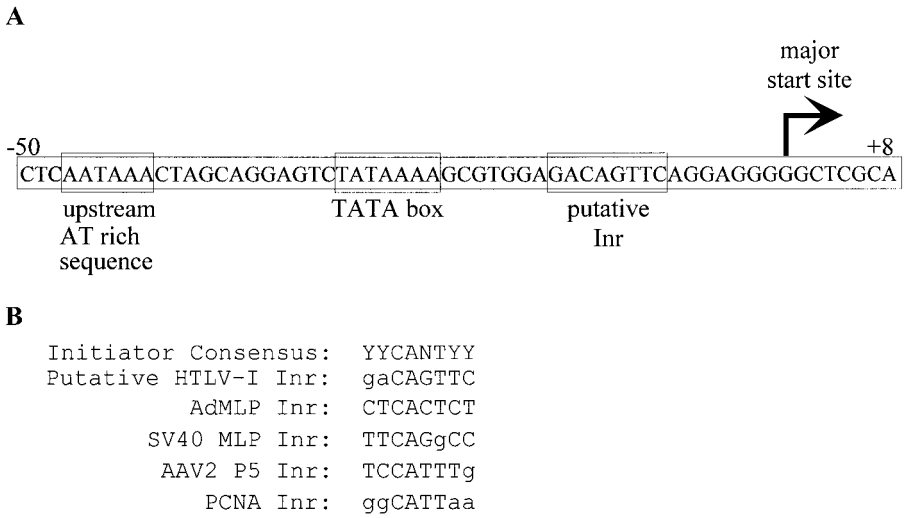

FIG. 1. Sequence of the HTLV-I initiator. (A) A schematic diagram of part of the HTLV-I genome. The virus utilizes the 5′ LTR as its sole promoter. Examination of the region of the LTR proximal to the major transcription start site identified an element with sequence similarity to an initiator (Inr). The position of the TATA box and an upstream AT-rich sequence are indicated. (B) Sequence alignment of the putative HTLV-I Inr element with several authentic initiators. The HTLV-I element matches the consensus sequence except at the two 5′ terminal bases.

moter region revealed a putative initiator (Inr) element (Fig. 1A), identified on the basis of homology to the consensus initiator sequence (Fig. 1B). Sequences of initiator elements vary considerably, but an approximate consensus sequence, $YYCA_{+1}NT/AYY$, has been identified (Bucher, 1990; Javahery *et al.,* 1994; Lo and Smale, 1996). Although the putative HTLV-I Inr contains the consensus CANT sequence and downstream pyrimidines, the upstream bases are purines. Latitude in identity of flanking bases has been reported for Inr elements as long as at least some are pyrimidines (Smale *et al.,* 1998). TATA boxes are often found ~30 bp upstream of cellular Inr elements, whereas the HTLV-I TATA box is only 14 bases upstream. Located ~30 bp upstream from the consensus initiator sequence, however, the HTLV-I promoter does contain an AT-rich sequence that functions as the poly A addition site in the 3′ LTR (Fig. 1A). Both the Inr sequence and the upstream AT-rich sequence are perfectly conserved between the HTLV-I and HTLV-II LTRs. These observations led us to test the hypothesis that this Inr-like sequence might play a biologically relevant role in HTLV-I transcription regulation.

The putative HTLV-I initiator exhibits specific protein binding

Initiator elements are thought to function in part by binding a transcription factor and nucleating the assembly of a basal transcription complex. To investigate the ability of the putative HTLV-I Inr to bind to a cellular protein, an electrophoretic mobility shift assay (EMSA) was employed using an oligonucleotide probe encompassing the Inr (Fig. 2A). Two prominent complexes formed on this probe (lane 2). Excess of the unlabeled "self" competitor reduced formation of the slower-migrating complex 1 (lane 3). An oligonucleotide containing a 3-bp mutation that disrupted the crucial CA dinucleotide of the Inr failed to compete for either complex (lane 4). To extend these findings, a direct binding assay was performed using the mutant Inr oligonucleotide as probe. The specific protein complex was not observed on the mutant Inr probe (lane 6). These results demonstrate that complex 1 forms specifically on the Inr probe, whereas the faster migrating complex 2 is non-specific. Using HeLa nuclear extracts fractionated on a phosphocellulose column, EMSA experiments demonstrated that the specific complex 1 was assembled with protein from a fraction eluting at 300 mM NaCl (Fig. 2B, lane 4).

SRF is part of the Inr binding complex

To examine the identity of initiator-binding proteins, competition and antibody shift EMSAs were performed (Fig. 3A). The probe formed two complexes with HeLa cell extracts (lane 2), identified as complex 1 and complex 2. An unlabeled oligonucleotide containing the HTLV-I Inr wild-type sequence competed complex 1 (lane 2), whereas a mutant oligonucleotide did not (lanes 3 and 4). An oligonucleotide containing the PCNA initiator sequence failed to compete with either complex (lane 5), suggesting that the PCNA and HTLV-I initiators have different binding specificities. Since YY1, E2F, and SRF have been among the proteins previously identified as components of Inr binding complexes (Seto *et al.,* 1991; Grueneberg *et al.,* 1997), antibodies specific for these proteins were tested for the ability to shift complex 1. Although YY1 antibody had no impact on the complex (lane 6), SRF antibody altered the position of complex 1, increasing its mobility (lane 8). Antibody to E2F also failed to alter the mobility of complex 1 (data not shown). To extend this result, the effect of SRF antibody on phosphocellulose column fractions of HeLa extract was

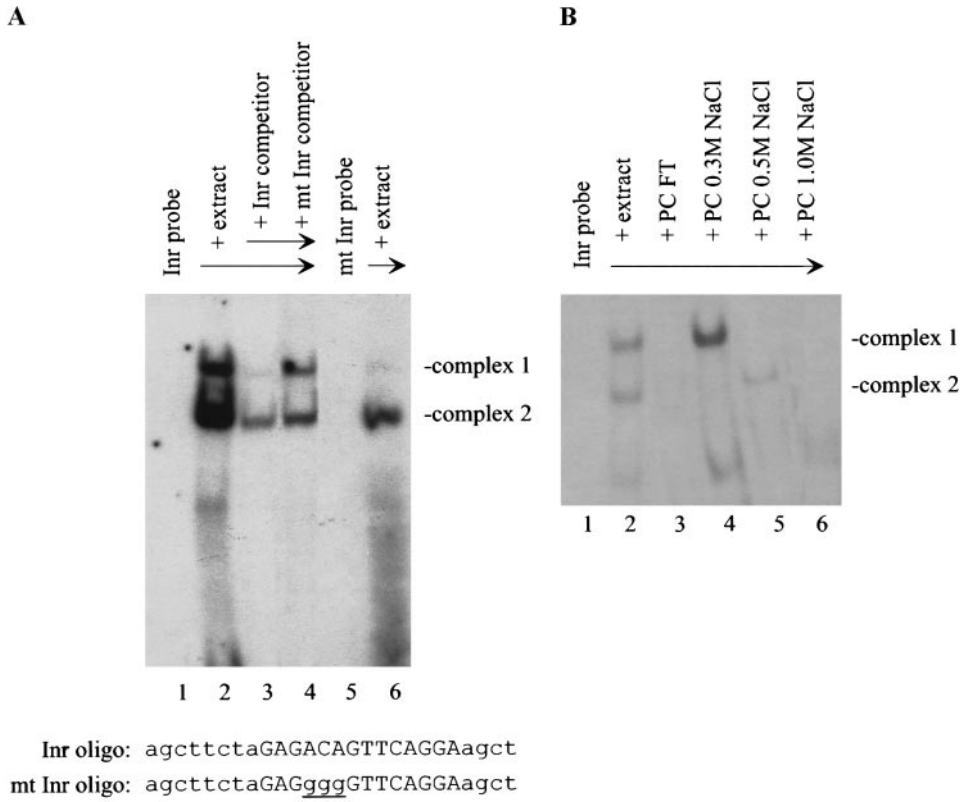

FIG. 2. Specific protein binding to the HTLV-I Inr and identification of a non-binding Inr mutant. Gel shift assays were performed to characterize the protein complex that forms on the putative HTLV-I Inr element. (A) Incubation of HeLa nuclear extract (lanes 2, 3, 4, and 6) with the Inr probe (lanes 1–4) resulted in two complexes (lane 2). The upper complex (complex 1), but not the lower (complex 2), was specific based on competition with unlabeled competitors corresponding to the wild-type Inr (lane 3) or an Inr mutant (lane 4). A direct binding assay was performed to test the Inr mutant directly (lanes 5 and 6). The labeled Inr mutant probe failed to promote formation of the specific complex 1 (lane 6). The sequences of the wild-type and mutant Initiators are shown below. (B) To characterize the specific complex further, gel shift assays using phosphocellulose (PC)-fractionated HeLa extracts were performed. The non-specific band was found in the fraction eluted with 0.5 M NaCl (lane 5), and the Inr-specific band was found in the fraction eluted with 0.3 M NaCl (lane 4). The bands were not detected in the flow through (FT) fraction (lane 2) nor in the fraction eluted with 1.0 M NaCl (lane 6).

examined by EMSA (Fig. 3B). Complex 1 was observed in the 0.3 M NaCl fraction, as seen previously, and that complex was shifted down by SRF antibody comparably to the whole cell extract (compare lanes 2 and 3 with lanes 6 and 7). In addition to confirming the presence of SRF in complex 1, these results imply that there are additional proteins in this complex and that competitive binding of the SRF antibody displaces the SRF from the complex.

The HTLV-I initiator is sufficient for gene expression in a heterologous system

To determine whether the putative Inr was sufficient to direct transcription in the absence of a TATA box, oligonucleotides representing wild-type (pHILuc) or mutant (pHImLuc) HTLV-I initiator sequences were cloned into a luciferase reporter. The ability of these constructs to promote luciferase expression was examined following transfection into HeLa cells (Fig. 4A). The construct containing the wild-type HTLV-I initiator (pHILuc) displayed an approximately sixfold increase in luciferase activity over the promoterless parent luciferase vector (pLuc). This activity was greater than that observed when the PCNA initiator was tested in a similar construct (data not shown). The initiator mutant (pHImLuc) exhibited threefold-reduced activity relative to the wild-type Inr construct. These experiments demonstrate that the HTLV-I Inr is sufficient as the sole promoter element to increase reporter gene expression, presumably through transcriptional activation, and that the Inr mutation that diminishes protein binding (Figs. 2A and 3) also reduces *in vivo* activity. Primer extension analysis to determine directly whether elevated luciferase expression resulted from transcriptional activation was not successful because of the low level of transcriptional activity stimulated by this initiator element (data not shown).

The HTLV-I initiator is not necessary for gene expression from the intact HTLV-I LTR

The data presented above demonstrate that the HTLV-I Inr sequence can function independently to confer transcriptional activity to a heterologous re-

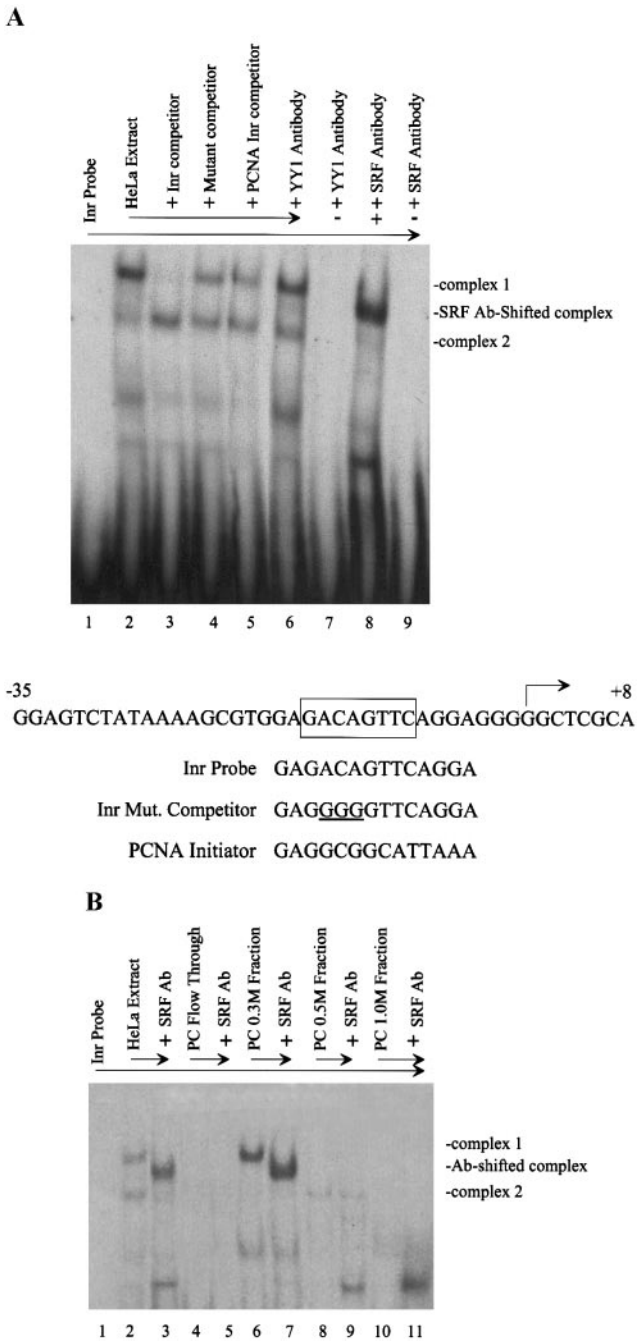

FIG. 3. SRF is a part of the specific Inr binding complex. (A) To establish the identity of protein(s) in complex 1, HeLa cell extract was incubated with the Inr probe and specific oligonucleotide competitors or antibody. Complex 1 formed with HeLa cell extract (lane 2) and was competed by self (lane 3). It was not competed with the Inr mutant oligo or with the PCNA initiator sequence (lanes 4 and 5). YY1 antibody failed to shift the complex or to bind the probe in the absence of cell extract (lanes 6 and 7). SRF antibody shifted the complex down (lane 8) and did not bind the probe in absence of cell extract (lane 9). (B) SRF antibody shifts the 0.3 M phosphocellulose fraction. HeLa cell extract fractionated over a phosphocellulose column was incubated with labeled Inr probe. Complexes 1 and 2 formed with HeLa cell extract as seen previously, and complex I was shifted by SRF antibody as previously (lanes 2 and 3). No specific complex was seen in the flow-through, 0.5 or 1.0 M fractions (lanes 4, 5, 8, 9, 10, and 11). Complex 1 was formed with the 0.3 M fraction (lane 6) and was shifted down with SRF antibody (lane 7) similarly to the whole cell extract (compare lanes 2 and 3 to lanes 6 and 7).

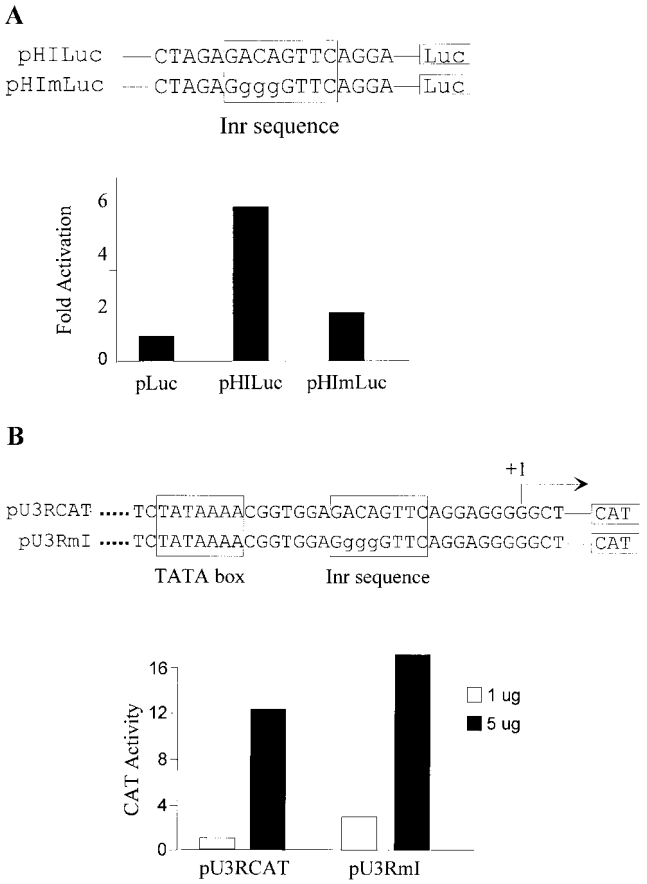

FIG. 4. The HTLV-I Inr is sufficient but not necessary to activate reporter gene expression. (A) HeLa cells were transfected with pLuc, pHILuc, or pHImLuc (1 μg per plate). Each transfection included 1 μg of pSV β-Gal as an internal control. Luciferase assays were performed to measure promoter activity. All results have been corrected for transfection efficiency by dividing luciferase units by β-Gal units. The corrected activity of pLuc was set to 1, and corrected values for pHILuc and pHImLuc were normalized to pLuc to obtain fold activation. (B) Basal transcription of the wild-type (pU3RCAT) and Inr mutant (pU3RmI) LTRs was evaluated by transfection assays. Proximal promoter sequences of constructs are depicted above. Dotted lines indicate continued upstream LTR sequence. HeLa cells were transfected with 1 or 5 μg of reporter. Each transfection included 1 μg of pGL3Luc-Basic as an internal control. All results have been corrected for transfection efficiency by dividing CAT units by luciferase units. The results shown are representative of at least three independent experiments.

porter construct. To determine the role of this element in transcription control of the HTLV-I LTR, the previously described 3-bp Inr mutation was introduced into the LTR by site-directed mutagenesis (pU3RmI). The effects of this mutation on the transcriptional activity of the LTR were measured by transfection and reporter CAT assay (Fig. 4B). Basal activity of the Inr mutant was similar to that of the wild-type LTR, indicating that the initiator sequence is not necessary to promote transcription within the context of the complete LTR, a reasonable result given the large number of transcription factor binding sites extant in the LTR and the activated state of the cultured cells.

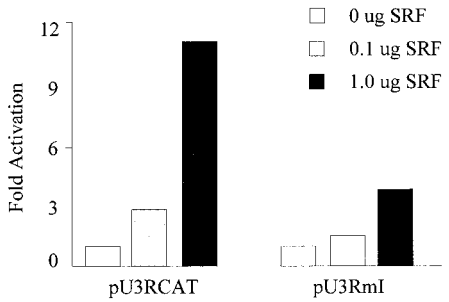

FIG. 5. SRF augments activity of the wild-type Inr. HeLa cells were transfected with either an HTLV-I promoter containing the wild-type Inr sequence (pU3RCAT) or one with the 3-bp Inr mutation sequence (pU3RmI) alone or in combination with an SRF expression plasmid (0.1 or 1 μg). Each transfection included 1 μg of pGL3-Luc control as an internal control. Corrected CAT activity is shown as fold activation over the CAT activity measured in the absence of SRF. At 1 μg SRF, the wild-type promoter was about fourfold more active than the mutant promoter. The results shown are representative of three independent experiments.

Exogenous SRF augments transcription

Since SRF was identified as a component of the Inr binding complex, it was important to examine the role of SRF in the function of the initiator *in vivo.* The wild-type promoter construct was transfected into HeLa cells with increasing concentrations of an SRF expression vector (Fig. 5). An 11-fold increase in promoter activity was observed upon addition of 1 μg SRF. In contrast, transfection of the mutant HTLV-I LTR (pU3RmI) containing the 3-bp mutation in the Inr that diminished specific complex formation (Fig. 2) resulted in reduced activation by SRF. The residual SRF-induced activity of pU3RmI may result from other SRF responsive sites in the LTR (Pastorello and Marriott, unpublished results). These data suggest that the intact initiator element confers increased responsiveness to SRF, presumably through formation of complex 1 (Fig. 2).

The initiator sequence influences transcription start site selection

To examine the viral transcription start sites in infected cells, RNA was isolated from the HTLV-I-infected lymphocyte cell line, HuT-102, which contains a transcriptionally active, integrated provirus. The transcription start site was mapped using primer extension and compared to transcripts resulting from cells transfected with pU3RCAT or pU3RmI with or without Tax. RNA from HuT-102 cells and cells transfected with pU3RCAT plus Tax showed a similar pattern of heterogeneous start sites around +1 and some initiation at −14 and −16 (Fig. 6, lanes 1 and 2). In the presence of Tax, transcripts originating from pU3RmI (Inr mutation) displayed increased use of start sites at +3, +5, and +6, whereas use of the authentic +1 start site decreased (lane 3). In the absence of Tax, pU3RCAT and pU3RmI showed major transcript initiation at positions +1, +2, and +3 (lanes 5 and 6), and pU3RmI still showed increased usage of +5 and +6 start sites (lane 6), indicating that the effect of the Inr mutation on the transcripts originating at +5 and +6 was independent of Tax. From these results, Tax appears to direct the minor −14 and −16 transcripts as well as increasing the overall level of transcription. Since pU3RmI displayed reduced initiation from the authentic +1 start site and increased use of adjacent start sites, the Inr contributes to correct initiation. Interestingly, in a model promoter construct containing an Inr positioned between the TATA box and the transcription start site, as organized in the HTLV-I LTR, no transcripts originating within the Inr element were observed; rather the Inr served to increase transcription at the +1 site (O'Shea-Greenfield and Smale, 1992). The position and function of the HTLV-I initiator recapitulates those findings within the context of a natural promoter.

DISCUSSION

Intimation that the promoter of HTLV-I might contain an initiator element arose first from studies showing that mutation of the HTLV-I TATA box did not dramatically reduce levels of transcription (Nicholas and Nevins, 1991) and subsequently from examination of sequence

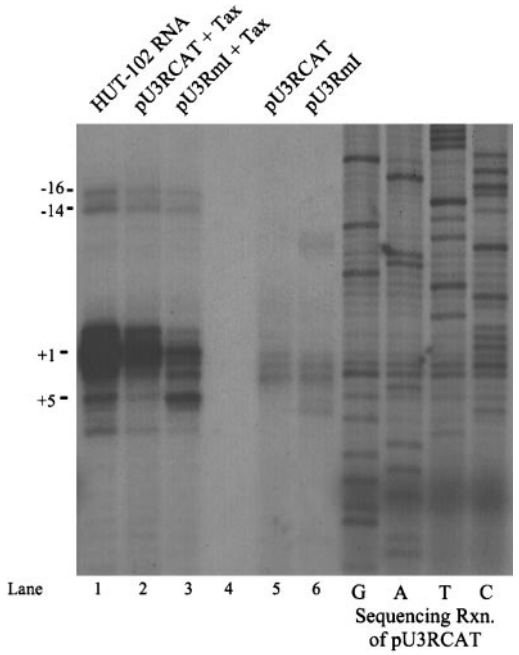

FIG. 6. The Inr element affects transcripts near the +1 start site. RNA was harvested from HuT-102 cells or from HeLa cells transfected with 10 μg pU3RCAT or pU3RmI, with and without 1 μg Tax. The HuT-102, pU3RCAT + Tax, and pU3RmI + Tax lanes each contained of 20 μg of RNA (lanes 1–3). The pU3RCAT and pU3RmI lanes each contained 100 μg of RNA (lanes 5 and 6). Each primer extension reaction utilized a common radiolabeled oligonucleotide. The quality of each RNA preparation was similar, as judged by agarose gel analysis (data not shown). The sequencing reaction utilized the same oligonucleotide primer used for primer extension allowing determination of the exact base from which transcripts originated.

homology. The putative HTLV-I Inr contains six of eight bases present in the consensus initiator, including the entire Inr core sequence (5′-CANT-3′) and the downstream pyrimidine residues. Although 5′ flanking pyrimidines are absent, it is not uncommon for functional Inrs to vary in one or more flanking residues from consensus (Smale *et al.,* 1998), and the most critical residues, an A at +1 and a T at +3 are intact in the HTLV-I LTR.

The atypical position of the putative HTLV-I initiator may affect its properties. Most Inrs in TATA/Inr promoters are situated 25 bases downstream of the TATA box. In this arrangement, the two elements cooperate to direct transcription from the same base, generally A of the Inr CANT sequence. The putative HTLV-I Inr is positioned between the TATA box and the TATA-directed start site. To our knowledge, this configuration of basal elements has not been previously reported in a natural promoter and might recruit a collection of general transcription factors with unique properties.

Several cellular proteins have been reported that bind different viral Inrs, recruiting the general transcription machinery. A general transcription factor, TFII-I, has been shown to bind the Inr of the adenovirus major late promoter (Roy *et al.,* 1991, 1997). The Inr of the SV40 major late promoter binds E2F, a transcription factor important in regulating cell cycle progression (Kraus *et al.,* 1996), and the Inr of the adeno-associated virus type 2 P5 promoter has been shown to bind Ying-Yang 1 (YY1), a Kruppel-related transcriptional activator/repressor (Seto *et al.,* 1991; Kyostio *et al.,* 1995). The two largest TFIID sub-units, TAF 150 and TAF 250, have also been implicated in transcription directed by Inrs (Kaufmann *et al.,* 1998; Verrijzer *et al.,* 1995; Oelgeschlager *et al.,* 1996; Chalkley and Verrijzer, 1999). In our experiments, the putative HTLV-I Inr element specifically binds a protein complex that elutes from a phosphocellulose column at 300 mM NaCl, and an SRF antibody shifts the position of the specific protein complex in this fraction. Antibodies to YY1 failed to affect migration of the Inr gel shift complex, as did antibodies to E2F (data not shown). Although these results implicate SRF in the binding to the HTLV-I initiator element and eliminate a role for YY1 and E2F, other proteins remain Inr-binding candidates. In light of a report that TFII-I, a known initiator-binding protein (Manzano-Winkler *et al.,* 1996; Roy *et al.,* 1991) interacts with SRF (Grueneberg *et al.,* 1997), its role will be particularly interesting to examine.

Transcription start sites mapped in this study from the wild-type HTLV-I LTR integrated in HuT-102 cells and from transfected pU3RCAT plasmid are concordant with previous reports. This analysis confirmed the +1 start site and demonstrated that Tax increases transcription initiation at the +1 site as well as two additional sites, −14 and −16. The −14 and −16 start sites map near the Inr located at −13 to −8 but do not appear to be directed by it. In a synthetic construct, addition of a Inr between a TATA element and the TATA-directed start site resulted in enhanced transcription from the TATA-directed site rather than transcription initiation within the Inr (O'Shea-Greenfield and Smale, 1992). Our results with a naturally occurring sequence from the HTLV-I LTR affirm those data; the HTLV-I Inr-enhanced transcription from the TATA-directed start site but did not direct initiation from an internal site. Moreover, mutation of the Inr element in the LTR changed the preference of start sites. By contributing to correct start site positioning, the initiator may help insure integrity of the HTLV-I genome during viral replication.

It is possible that the Inr plays a role in HTLV-I transcription only at specific stages of the viral life cycle. T cells, the natural reservoir for HTLV-I, are generally in a quiescent state in which transcription factors are limiting and housekeeping genes, often regulated by initiator elements, are preferentially transcribed. In this context, the virus may utilize the initiator-like element to launch basal transcription. Production of the viral Tax protein from these initial rounds of basal transcription may allow subsequent activation of cellular genes, converting the cell to an activated state and allowing direction of transcription from the primary TATA box.

The results reported here have identified an element with a high degree of identity to a transcriptional initiator element. The ability of this element to bind a specific protein complex containing SRF and to support transcription independently *in vivo* is consistent with the function of this element as an Inr. As future studies more completely dissect basal regulatory elements in the HTLV-I LTR, the role of the Inr in basal and Tax-activated transcription should be clarified.

MATERIALS AND METHODS

Plasmids

pU3RCAT has been previously described (Sodroski *et al.,* 1984). The following plasmids were purchased from Promega: pGL3-Luc Basic (a promoterless, enhancerless luciferase expression plasmid), pGL3-Luc Enhancer (a promoterless luciferase expression vector that contains one copy of the SV40 enhancer), pGL3-Luc Control (a luciferase expression vector under control of the SV40 promoter and enhancer), and pSV-β-galactosidase (a β-galactosidase expression plasmid for monitoring transfection efficiencies of mammalian cells).

The pU3RmI construct, containing a 3-bp mutation in the putative Inr element was generated by mega-primer site-directed mutagenesis (Barik, 1995). Briefly, a sense-strand oligo containing the mutation (5′-AAAAGCGTGGAGGGGGTTCAGGAGGGG-3′) was used as one primer in a PCR reaction. The anti-sense primer (5′-AGTATCCTCGAGGACTCT-3′) corresponded to a region of the pU3RCAT plasmid backbone. PCR, using pU3RCAT as the template, yielded a 453-bp PCR product, which was then used as a sense-strand "mega-primer" in a second round of PCR. This PCR reaction used an anti-sense

primer (5′-TGCCTCAAAATGTTCTTTACGATGCCATTGG-3′) corresponding to a different region of the pU3RCAT template. The PCR product was purified, digested with *Xho*I and *Hin*dIII, and cloned into pU3RCAT. The resulting construct was sequenced to ensure that only the desired mutation was introduced into the LTR. Inserting one of the following oligonucleotide cassettes (sense strand shown) into the HindIII site of pGL3-Luc Enhancer generated plasmids pHILuc and pHImLuc:

putative HTLV-I Inr (to make pHILuc)	5′-AGCTTCTAGAGACAGTTCAGGA-3′
mutated HTLV-I Inr (to make pHImLuc)	5′-AGCTTCTAGAGGGGGTTCAGGA-3′

These plasmids were sequenced to ensure that only one copy of the element was present and that it was in the correct orientation.

Gel shift assays

Gel shift probes were labeled with [^{32}P]α-dCTP using a Klenow fill-in reaction and gel purified. One nanogram of probe was used for each gel-shift reaction. For gel shifts using nuclear extract, the reaction also contained 1 μl of HeLa nuclear extract (6.2 μg/μl), 1 μg/μl of poly(dI/dC), 6 μl of 5× gel shift buffer [50 mM HEPES (pH 7.9), 20% Ficoll, 250 mM KCl, 1 mM EDTA (pH 8.0), 1 mM DTT], and 18 μl of buffer D [20 mM HEPES (pH 7.9), 100 mM KCl, 12.5 mM $MgCl_2$, 17% Glycerol, 1 mM EDTA (pH 8.0), 1 mM DTT]. Competitor DNAs were used at 100-fold molar excess.

Cells

HeLa cells were grown in DMEM medium supplemented with 10% fetal bovine serum. The human T cell line HuT-102, which contains an integrated HTLV-I provirus and produces infectious virus, was cultured in RPMI medium supplemented with 10% fetal bovine serum.

Transfections

HeLa cells were transfected by calcium phosphate precipitation as previously described (Connor *et al.,* 1993), and an internal control for transfection efficiency was always included. If the primary reporter was CAT, the internal control was pGL3-LucControl; if the reporter was a luciferase-based expression plasmid, the internal control was pSV β-Gal. Cells were harvested approximately 48 h posttransfection. CAT activity was assayed by a single phase extraction protocol (Seed and Sheen, 1988). Both luciferase and CAT assays used 25 μl cellular extract, and CAT assays were performed in duplicate. β-Gal assays were performed by the modified 96-well plate method using 50 μl of whole cell extract (Rosenthal, 1987). Sample absorbance was measured at 414 nm. A representative result of at least three experiments is shown.

Primer extension assays

RNA used for primer extension experiments was harvested either from transiently transfected HeLa cells or from untransfected HuT-102 cells. Briefly, 60-mm dishes of cells were lysed in guanidinium isothiocyanate solution [4 M guanidinium isothiocyanate, 20 mM sodium acetate (pH 5.2), 0.1 mM DTT, 0.5% Sarkosyl]. Cell lysates from replica plates were pooled, sheared, and purified by CsCl gradient centrifugation. Single-stranded oligonucleotides were labeled with ^{32}P to generate primer extension probes that were complementary to the non-coding strand of the DNA at a site ~50 bases downstream of the expected transcription start site. Each assay used 10–20 μg of total RNA from HTLV-I LTR constructs in the presence of Tax. For LTR constructs in the absence of Tax, 80–100 μg of RNA was required to detect HTLV-I transcripts. For each reaction, ^{32}P-labeled oligo probe was hybridized with an appropriate amount of RNA and extended using AMV reverse transcriptase (Promega). Products were analyzed on an acrylamide/urea sequencing gel. Primer extension reactions were performed in parallel with a sequencing reaction (Sequenase v2.0 DNA sequencing kit, Amersham) utilizing the same primer.

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